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Analysis of free and bound NADPH in aqueous extract of human placenta used as wound healer☆

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ABSTRACT

NADPH is an important biomolecule involved in cellular regeneration. The distribution of free and bound NADPH in aqueous extract of human placenta used as a potent wound healer has been analyzed. Quantification from fluorescence and immuno-affinity chromatography indicates that 75.1 ± 2.2% of NADPH present in the extract exists as free nucleotide or bound to very small peptides or amino acids whereas the rest remains bound to large peptides. Inability to dissociate the bound form of the nucleotide from the large peptides using urea or guanidium hydrochloride indicates that the binding is covalent. Identification of a fragmented mass of *m/z* 382.94 (nicotinamide + sugar + phosphate) from the NADPH-peptide conjugates supported the intactness of the nicotinamide moiety. Glutathione reductase assay indicated that 95.2 ± 3.5% of the total NADPH pool of the extract can act as cosubstrate of the enzyme. This indicates that while a major fraction of free NADPH of the extract is easily available for cellular processes, the rest can also function locally where the conjugated peptides are deposited.

1. Introduction

Traditional knowledge has guided the development of several modern medicines, scores of which require adequate scientific evaluation to ensure their safe use. An aqueous extract of human placenta is one such example that has been attributed with healing of wounds and find applications in post-surgical dressings, in the treatment of chronic non-healing wounds of diabetic patients and high degrees of burn injuries [1–6]. The extract is an important bio-stimulator and is used in the treatment of myopic and senile chorio-retinal dystrophies [7], infertility [8], vitiligo with topical melagenine [9], psoriasis [10,11], arthritis [12], cerebral arteriosclerosis [13] and in preventing recurrent respiratory infections [14]. Clinically tested over time, the potency of the extract has not yet been established on biochemical grounds. However, use of placental extract from animal sources in different preparations under various trade names is a worldwide practice.

Currently we are characterizing an indigenous preparation of an aqueous extract of human placenta used as a licensed drug for wound healing. Its manufacture is well standardized as has been demonstrated by chromatographic and spectroscopic methods [15]. Other findings are its anti-microbial property against commonly occurring pathological micro-organisms [16], ability for *in vitro* NO induction in mouse peritoneal macrophages [17] and ability to enhance cell adhesion [18]. Presence of fibronectin type III-like peptide, a basement membrane component, in the extract has been confirmed [19]. Partial amino acid sequence and presence of signatory RGD sequence required for cell adhesion in the fibronectin peptide indicate that it is derived from the 10th type III module of fibronectin [18,19]. NADPH, an important nucleotide involved in several cell signaling and cellular regeneration processes, is also present in the extract [20]. These properties are directly or indirectly supportive to wound healing activity of the extract.

During the course of investigation, it was observed that the isolated peptide mixture of the extract showed fluorescence emission that was similar to NADH or NADPH and unusual for a peptide constituted of standard amino acids. The extract is known to be devoid of NADH but contains NADPH [20]. Thus, some degree of association between NADPH and the peptides was speculated. Since the biological potency of free and peptide bound NADPH are likely to be different, we present an analysis of the distribution of this nucleotide.

Separation of free and bound fractions of a protein–ligand mixture is most conveniently achieved based on difference in molecular weight and solubility. These include, dialysis, ultrafiltration with membranes of required molecular weight cut-off range and determination of free fraction by cloud point extraction [21]. Peptides particularly the smaller ones, on the contrary, have molecular weight and solubility often similar to the ligands of low molecular weight. Thus, the methods outlined above are not suitable for

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peptide–ligand mixtures. Various other methods must therefore be employed to confirm the ratio of free to bound fractions in a peptide–analyte mixture.

Estimation of NADPH in biosamples can be done optically at 340 nm but is often interfered by the presence of other absorbing molecules. Fluorimetric determination of NADPH (ex: 340 nm; em: 450 nm) is fairly specific as except NADH, very few molecules fluoresce in that region and is of high sensitivity. NADPH and NADH can be simultaneously estimated by reverse phase HPLC monitoring at 340 nm even under impure conditions [22,23]. Probably the most reliable way to estimate NADPH is by glutathione reductase assay where absolute specificity of an enzyme for its substrate is employed [24]. Mass spectrometric analysis also confirms presence of NADPH in a sample but its quantification is difficult at least under impure conditions. Here we have used a combination of these methods as applicable.

2. Materials and methods

2.1. Placental extract

The drug house M/s Albert David Ltd., Calcutta, India, supplied human placental extract used as a licensed drug under the trade name 'Placentrex'. Preparation of the extract by a single hot and cold aqueous extract holding manufacturer's proprietary protocol has been described earlier [20]. The product was sterilized under saturated steam pressure of 15 psi at 120 °C for 40 min that is subsequently referred as heat treatment. The extract contains 1.5% (v/v) benzyl alcohol as preservative, which does not interfere with spectral profiles as presented here. For biological safety, the extract was routinely tested for HIV antibody and Hepatitis B surface antigen.

2.2. Reagents

NATA (*N*-acetyltryptophan amide), NADPH, NADH, bovine serum albumin (BSA, fraction V), ovalbumin, lysozyme (chicken egg white), Freund's adjuvant (complete and incomplete), anti-mouse IgG (whole molecule) alkaline phosphatase, DEAE-cellulose, nitrocellulose membrane, BCIP (5-bromo-4-chloro-3-indolyl phosphate), NBT (nitro blue tetrazolium), glutathione reductase (type II crude wheat germ, MES [2-(*N*-morpholino) ethansulfonic acid], amino acids and dialysis tubing (normal and benzoylated having 10 and 2 kDa cut-off range) were from Sigma, USA. CarboxyLink™ coupling gel (immobilized diaminodipropylamine) and EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, HCl] were from Pierce, USA. Pronase E (*S. griseus*) was from Merck, Germany. Millex-GV and 0.22 μm filter unit (Millipore, USA) were used for filtration of samples before optical analysis. Distilled water was passed through high performance reverse osmosis purification system (Arium 61315, Sartorius, Germany).

2.3. Production of antibody

Peptides being weakly immunogenic compared to proteins, the following procedure was standardized to raise antibody against placental peptides. Balb/c mice ($n = 10$) were primed *intra peritoneally* (i.p.) with an emulsion of placental peptides (1.0 mg/ml) in Freund's complete adjuvant (1:1, v/v). On the 21st day, animals were injected i.p. with a booster dose of the peptide emulsified in Freund's incomplete adjuvant. Another booster dose on the 7th day ensured increased peak of antibody production. After 7 days from the last injection, 0.5–0.8 ml of blood was drawn from the tail vein of the mice and serum was collected. Antibody was purified from the serum by fractionation with 50% ammonium sulfate and pellet resuspended with a volume equal to that of original serum sample followed by dialysis with 10 mM K-phosphate, pH 6.8. Finally the dialyzed sample was applied to 2-ml DEAE-cellulose column equilibrated with the same buffer. The column was washed with equilibration buffer when the antibody was eluted uncontaminated as unabsorbed fraction [25].

2.4. Isolation of placental peptides

2.4.1. Conventional method

The procedure was after Ref. [19] with minor modifications. Placental extract as supplied (20 ml) was dried in wide mouth glass beaker in vacuum desiccators over NaOH pellets at ambient temperature. The dried mass was reconstituted with 1 ml of water and centrifuged at 10 K rpm for 10 min. The yellowish liquid that was separated between the pellet and the floating fat layer was collected carefully and filtered under centrifugal force (avg. $2000 \times g$) by Centricon nylon membrane (YM-10, cut-off limit 10 kDa, Amicon, USA). The filtrate was dialyzed by benzoylated membrane against water with several changes to remove salts, very small peptides and amino acids, if any. Finally, 100 μl aliquots of the dialyzed fraction were passed through Sephadex G-15 spin column, thereby resulting in better purification [26].

2.4.2. Immuno-affinity

This protocol was pursued as the peptide pool purified by conventional method lead to aggregation during concentration of the extract and also upon storage. A 2-ml column was packed with CarboxyLink™ coupling gel. After settling for 30 min, it was equilibrated with five column volumes of coupling buffer (0.1 M MES, pH 4.7). Purified antibody (1–5 mg/ml in MES) was added to the gel slurry and gently mixed for several minutes. Immediately, EDC (0.5 ml from a stock of 120 mg/ml) was added to the matrix containing the antibody and the mixture was incubated for 3 h with mild shaking at 25 °C. After settling under gravity, the supernatant was drained off and the column was thoroughly washed with 1 M NaCl [27]. The column was then equilibrated with 0.1 M Na-phosphate, pH 7.5. Four-fold concentrated placental extract, containing approximately 150 mg/ml of solid matters, was applied to the column and was washed with 20 volumes of the same buffer. Elution of the peptides was initiated with 0.1 M gly-HCl, pH 2.2. Eluted fractions were followed at $A_{280\,nm}$, fluorescence intensity (ex: 340 nm; em: 450 nm) and by dot blot. The distribution of the bound *versus* unbound fluorescence fractions of the immuno-affinity column as applied under the experimental conditions stated above remained unaltered when the amount of sample applied was half or double. This ensured that there was no overloading of the column. Considering that the NADPH fluorescence (ex: 340 nm; em: 450 nm) applied to the column was 100%, total recovery of the same fluorescence from the unabsorbed fractions and the fractions recovered at low pH accounted for nearly 98.5%. Thus, nonspecific binding being insignificant, no correction was made.

2.5. Dot blot (immuno-blot)

Immunological cross reactivity was checked by dot blot experiments. Fractions from immuno-affinity column were spotted on nitrocellulose membrane strips, dried completely and incubated with PBS containing 0.1% Tween-20 and 5% skim milk at 4 °C for 18 h. The strips were washed four times with PBS containing 0.5% Tween-20 for 15 min each. Following this, the strips were incubated with mouse anti-sera of placental peptides that was diluted 1:1000 in PBS containing 0.5% Tween-20 and 1% skim milk with constant shaking for 2 h. Excess antibody was removed by washing as stated. The blots were then incubated with alkaline phosphatase conjugated anti-mouse IgG with constant shaking for 1 h. This was followed by washing thrice with 0.5% Tween-20 in PBS, 15 min each. The final washing was carried out solely in PBS for 15 min. Alkaline

phosphatase activity was revealed by color development using a solution of BCIP and NBT in 0.1 M Tris–HCl, pH 8.8. All reactions were carried out at 25 °C [28,29].

2.6. Spin column

A column of Sephadex G-15 (Amersham Biosciences, Sweden) (1-ml), equilibrated with 10 mM Na-phosphate; pH 7.5, was centrifuged at 2000 rpm for 2 min. Samples containing placental peptides (100 μl) were loaded on the pre-spun column and were centrifuged under identical conditions. The peptide was eluted in the void volume while small molecules were retained in the column. Recovery was 95–100% [26].

2.7. TLC

NADPH, amino acids and their conjugates produced after heat treatment, if any, were separated on Silica gel 60 F_{254} TLC plates (6 cm × 8.5 cm, Merck, Germany) using the following solvent systems: (a) ethanol:water (90:10, v/v), (b) ethanol:ethyl acetate:water (70:20:10) and (c) ethanol:acetic acid:water (80:10:10). Chromatograms were developed at 25 °C and spots were viewed under short wavelength UV irradiation (254 nm, Mineralight lamp, Model No. UVGL-25, UVP, Upland, C.A. 91786, USA).

2.8. Pronase digestion and RP-HPLC

Placental peptides (2 mg/ml) were treated with pronase (50:1, w/w) in 10 mM Na-phosphate, pH 7.5 at 37 °C for 72 h. Pronase being a protease of broad specificity; placental peptides were reduced down to the level of amino acids. The resultant incubate was analyzed using a reverse phase C_{18} μ-Bondapak (7.8 mm × 300 mm, 125 Å, 10 μm, Waters) column that was equilibrated with 0.01 M K-phosphate, pH 7.5 at a flow rate of 1 ml/min. Under such conditions, nucleotides associated with the peptide and their derivatives were separated [30]. Aliquots of 30 μl were applied and eluted under isocratic conditions with the equilibrating buffer. Elution of components was monitored at $A_{340\,nm}$. The peaks obtained were collected separately and subjected to mass spectrometric analysis.

2.9. Mass spectrometry

Peptides separated by HPLC were dissolved in 50% acetonitrile. A Q-Tof micro (Micro mass) mass spectrometer under positive ionization mode was used for analysis at a desolvation temperature of 200 °C. Argon as collision gas at 2 kg/cm^2 having collision energy of 10 eV was applied and micro channel plate detectors were used.

2.10. Estimation of NADPH

Standard NADPH solution in water was prepared using $\varepsilon^{M}_{340nm} = 6.27 \times 10^3$. A calibration curve was constructed with 0–75 μM of NADPH against emission intensity (ex: 340 nm; em_{max}: 450 nm) where linear dependence was observed ($R^2 = 0.992$, R^2 = regression coefficient). The points of the curve were the average of three sets where maximum variation observed was ±3%. Concentrations of NADPH in test samples were calculated based on its intensity at emission maxima (ex: 340 nm) assuming absence of quencher in the samples.

Concentration of NADPH, acting as cosubstrate of glutathione reductase, was assayed spectrophotometrically [24]. A reference assay mixture contained 18.76 μM of standard NADPH, 2.0 μM of oxidized glutathione and 11.8 U of glutathione reductase in 1 ml of 0.1 M K-phosphate, pH 7.5. The reaction was followed for 30 min at 410 nm. NADPH content of the extract was estimated by replacing an equivalent volume of standard NADPH with the extract. Alternately, NADP being nonfluorescent, oxidation of NADPH to NADP by the enzyme was also followed spectrofluorimetrically (ex: 340 nm; em: 425 nm). The later assay is more reliable because organic substances having absorbance at 340 nm do not interfere with fluorescence measurements as long as no 'inner filter effect' is observed.

2.11. Estimation of peptide

Reagents for quantifying proteins often give erroneous results for peptides. To avoid this, concentration of placental peptides was determined from $A_{225\,nm}$, which is contributed primarily from polypeptide links [31]. Standard solutions of BSA, ovalbumin and lysozyme were made in 10 mM Na-phosphate, pH 7.0 using $\varepsilon^{1\%}_{280nm}$ of 6.6, 7.5 and 24.0 respectively. The solutions were filtered through 0.22 μm filter membrane to remove particulate matters. Absorption of buffer was 0.211. No optical values greater than 1.5 was accepted. Correlation between $A_{225\,nm}$ *versus* concentration (5–30 μg/ml) of proteins was linear (corresponding regression coefficient, *R*, were 0.978, 0.984 and 0.996). The slopes of the curves were 0.0089, 0.0102, and 0.0177 $A_{225\,nm}$/μg of protein. A line having the average of the slopes of 0.012 was used for determination of peptide concentration that was found to be 3.16 ± 0.34 mg/ml ($n = 2$).

2.12. Other methods

Fluorescence measurements were done using a Hitachi F 4500 or F 3010 instrument having ex/em slit widths of 5 nm each. Optical absorbances were recorded with a Specord 200 spectrophotometer (Analytic jena, Germany). Balb/c mice (25 ± 5 g, either sex, 12–14 weeks) were obtained from in-house facility. All animal experiments were done following local animal ethics legislatures.

3. Results

3.1. Fluorescence of placental peptides

The fluorescence spectrum of placental peptides purified by conventional method showed strong emission between 400 and 550 nm when excited at 340 nm. This profile is very similar to that of the extract itself and bears resemblance to that of NADH or NADPH (Fig. 1A) [20]. Normalized spectrum of peptides and NADPH revealed that the em_{max} of the peptides had a blue shift of 22.0 nm compared to NADPH (463.1 *vs* 441.0 nm).

The emission patterns of the peptides, NATA and NADPH between 300 and 600 nm when excited at 295 nm were also compared. Emission zones for NATA (300–475 nm having em_{max} at 359.4 nm) and NADPH (400–570 nm having em_{max} at 461.2 nm) were essentially non-overlapping. If placental peptides were constituted of standard aromatic amino acids, its emission should be limited up to 450 nm like NATA. Further, its emission maxima had a red shift of 5.0 nm, i.e. 359.4 *versus* 364.4 nm as compared to NATA. In addition, it has a shoulder centered at 450.0 nm (Fig. 1B). The excitation spectrum of placental peptides where emission was fixed at 441 nm showed strong excitation between 300 and 400 nm having ex_{max} at 355.4 nm. This has resemblance to NADPH having ex_{max} at 344.2 nm. Placental peptides also showed strong excitation below 300 nm because of aromatic amino acid residues (Fig. 1C). Since the extract is devoid of NADH [20], these results collectively suggest that a fraction of NADPH probably remains associated with placental peptides.

Fluorophoric heterogeneity of placental peptides has been demonstrated by altering its excitation wavelength from 290 to

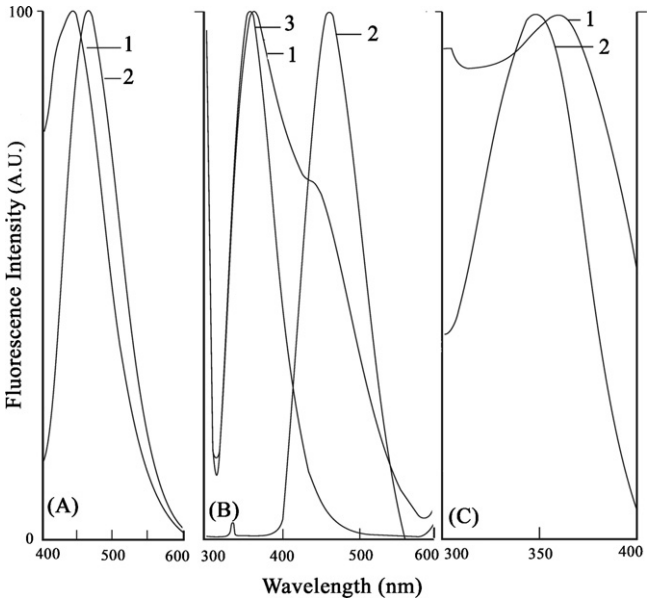

Fig. 1. (A) Fluorescence emission spectra of placental peptides (1) and NADPH (2) (ex: 340 nm). Em_{max} of the peptides has a blue shift of ~22 nm compared to NADPH. (B) Emission spectra of the peptides (1), NATA (2) and NADPH (3) (ex: 295 nm). The em_{max} of NATA and the first peak of the peptides roughly overlap with ~5 nm red shift for the peptide. The second unresolved peak of the peptides roughly corresponds to NADPH emission. (C) Excitation spectra of NADPH and the peptides (em: 441 nm). The ex_{max} of the peptides (355 nm) shows a blue shift of ~11 nm as compared to NADPH (344 nm).

310 nm at an interval of 2 nm. The result shows that while the emission intensity at 360 nm has been gradually reduced to 16%, corresponding emission at 450 nm remained unchanged (Fig. 2). This is in agreement with the fact that while the extinction coefficient of tryptophan is reduced by approximately 400% between 290 and 310 nm, the absorption profile of placental peptides between

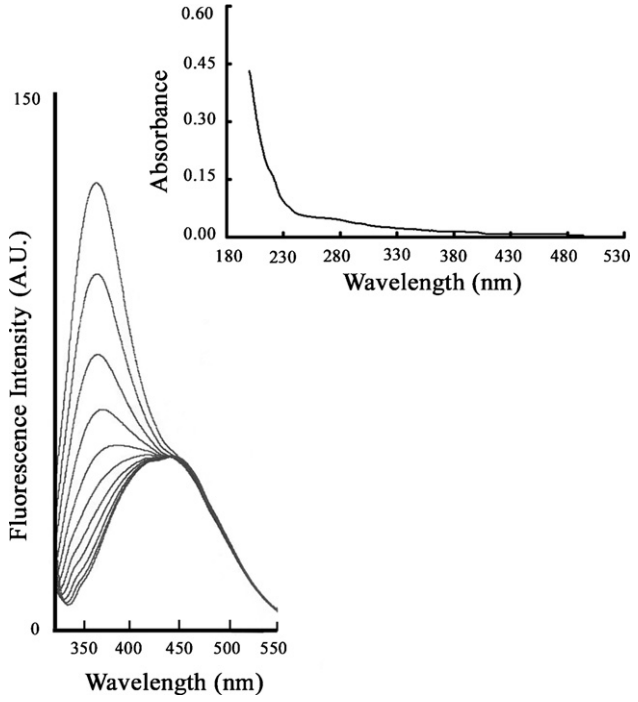

Fig. 2. Emission spectra of placental peptides while exciting between 290 and 310 nm at an interval of 2.0 nm. Inset: absorption spectra of placental peptides between 180 and 500 nm. Concentration of peptide was 35 μg/ml.

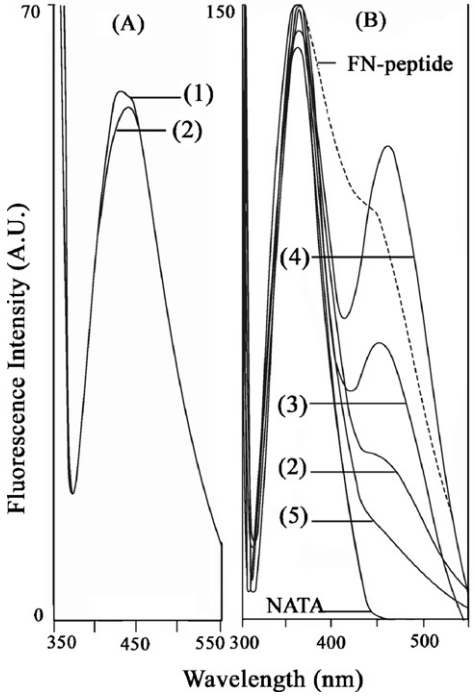

Fig. 3. (A) Emission spectra of placental peptides (ex: 340 nm) without any treatment (1) or after treatment with 8 M urea for 2 h or 16 h at 25 °C and passing through 'spin column' (2). A blue shift of 3–5 nm of em_{max} accompanied by 98 ± 2% retention of emission in presence of chaotropic agents is indicated. (B) Reconstruction of placental peptide-like profile, as indicated in the figure, by addition of 5 (2), 10 (3), 20 (4) and 30 (5) μl of NADPH (3.85 mM stock) to NATA (ex: 295 nm).

the same spectral zone remains low but marginally changed (Fig. 2, inset) [15].

3.2. Dissociation and reconstitution of peptide–NADPH conjugate

To assess the nature of binding between NADPH and placental peptides, the conjugate was treated with 8 M urea or 6 M guanidine, HCl at 25 °C in presence of 10 mM Na-phosphate, pH 7.5 for 16 h. The resulting incubate was passed through Sephadex G-15 'spin column' to remove free nucleotide, if any, and the eluent was subjected to emission scan (ex: 340 nm). It was observed that except a 3–5 nm blue shift of em_{max}, emission intensity was nearly identical compared to a control set where the peptides were not treated with the denaturants. Thus assuming that recovery of peptide from the 'spin column' was quantitative; no NADPH like fluorophore could be dissociated from the peptides indicating a strong association between the two (Fig. 3A).

Fluorescence property of a molecule containing non-interacting fluorophores should be additive. However, individual contributions of bound fluorophores may differ from that of the free fluorophores because fluorescence is highly sensitive to the polarity of the environment [32]. The emission spectrum of NATA was normalized with that of the placental peptides at respective emission maxima after exciting at 295 nm. NADPH was gradually added to NATA with the expectation that at a certain concentration, the emission spectra of placental peptides could be reconstructed as the emission zones of NADPH and NATA do not overlap when excited at 295 nm. It was observed that with increasing concentration of NADPH in NATA, a distinct peak having em_{max} at 460 nm appeared. In case of placental peptides with comparable intensity at 364.6 nm, the second emission appeared as a shoulder with an emission maxima centered around 450 nm (Fig. 3B). Thus, it is indicative that the environment of NADPH in placental peptides are different from its free state.

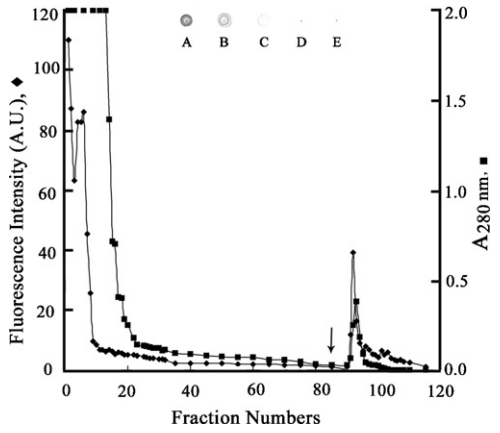

Fig. 4. Immuno-affinity chromatography of peptides from placental extract. The chromatogram shows that ~78.2% of NADPH is eluted in unbound fractions while the remnant is co-eluted with peptides. The arrow indicates application of elution buffer of pH 2.2. The apparent impression of separation of at least two components in the unabsorbed fractions is due to the difference of sensitivity of the parameters used to monitor the fractions i.e. fluorescence and absorbance, as a result two different scales of measurements were used. Inset: immunological cross reactivity between anti-sera of the purified peptide and placental peptides (A), bound fraction peak (B), pooled unbound fractions (C), lysozyme (D) and mouse serum (E) are indicated.

3.3. Immuno-affinity chromatography

The extract as supplied was directly applied to a column packed with placental peptide antibody coupled matrix. Developed profile was similar to a typical immuno-affinity chromatogram [33]. It shows that 75.1 ± 2.2% of the total fluorescence emission (ex: 340 nm; em: 450 nm) was eluted as unabsorbed fraction while the rest was co-eluted with the peptides (Fig. 4). This clearly indicated that a part of NADPH in the extract remains bound to placental peptides and cannot be dissociated under normal experimental conditions. Moreover, the peak obtained for the bound fraction showed low absorption at 280 nm of 0.383 as expected for the peptide. Presence of placental peptides in the bound fraction was confirmed by dot blot experiments where placental peptides purified by conventional method and lysozyme served as positive and negative controls respectively (Fig. 2, inset). Trace amount of peptides was observed in the unbound fraction from the cross reactivity of dot blot and had been neglected. This was not due to overloading of the sample in the column. Relative distribution of NADPH fluorescence appearing as free and bound to peptides has been mentioned in Table 1. Quenching of NADPH fluorescence by 1.82-fold at pH 2.2 compared to pH 7.5 was considered.

Table 1
Distribution of NADPH and peptide fraction in placental extract preparation.

Molecule	% Occurrence
(A) Total NADPH	100
Free NADPH	75.1 ± 2.2
NADPH bound to peptide fraction	23.8 ± 1.1
(B) Total peptide fraction	100
Free peptide	69.5 ± 1.5
NADPH bound peptide	30.5 ± 1.5
(C) Total peptide fraction–NADPH conjugate Sensitive to glutathione reductase	100
(i) Spectrophotometric estimation	34.4 ± 3.0
(ii) Spectrofluorimetric estimation	95.2 ± 3.5

3.4. Distribution of NADPH

In absence of NADH, emission from the test sample between 400 and 550 nm comes exclusively from NADPH or it's derivatives [20]. Results presented so far demonstrated that NADPH exists as free or low molecular weight conjugates. In addition, it remains bound with placental peptides. To distinguish this, placental extract was passed through Sephadex G-15 'spin column' and the fluorescence recovered in the eluent was considered as peptide bound NADPH. Similarly, distribution of NADPH in immuno-affinity chromatography in unabsorbed and absorbed fractions was considered as free or bound to amino acids and bound to peptides. In a separate set of experiments using purified peptides, its peptide and NADPH content were determined from absorbance at 225 nm and fluorescence at 435 nm. It shows that 33% of NADPH remains as free or bound to amino acids and 76% of placental peptides remain as free. The rest portions of the nucleotide and peptides remain as conjugate (Table 1).

3.5. Quantification of NADPH

3.5.1. Spectrofluorimetry

The placental extract demonstrated linear dependency of fluorescence intensity (ex: 340 nm; em: 450 nm) with concentration under the experimental conditions (R^2 = 0.977). A computation with the standard NADPH calibration curve, the nucleotide content of the extract was found to be 82.91 ± 38.5 μM i.e. 69 ± 32 μg/ml (number of batches, n = 28). Barring the extreme values occurring in some batches (n = 8), 53.19 ± 8.37 μM i.e. 44.33 ± 6.98 μg/ml of NADPH was estimated in the extract, which is the most widely occurring value tested so far.

3.5.2. Enzyme assay

Depletion of NADPH content was followed from decrease in absorbance at 340 nm as well as the characteristic fluorescence emission using glutathione reductase. Under the conditions of spectrophotometric assay, it was observed that 85.64 ± 1.36% of standard NADPH corresponding to 15.81 μM, was oxidized by glutathione reductase as cosubstrate (n = 2). When standard NADPH was replaced by 0.05 ml of the 20-fold concentrated extract, at the end of 30 min when enzymatic conversion was supposed to be complete, 34.4 ± 3.0% of absorption was decreased that corresponded to 2.91 μM of NADPH (n = 2). Since organic molecules of the extract absorbing at 340 nm interfered, the estimation was less accurate.

Fluorescence emission (ex: 340 nm) from the extract originate exclusively from NADPH, and thus, loss of emission intensity by glutathione reductase was directly related to the oxidation of the nucleotide. When 0.05 ml of the 20-fold concentrated extract was applied to the enzyme under assay conditions, ~95% loss of emission at 440 nm was observed. A standard NADPH solution of comparable fluorescence intensity showed 98% reduction of emission (n = 5) (Fig. 5A and B).

3.6. Nonspecific reactions of NADPH with proteins and amino acids

Nicotinamide nucleotides bind noncovalently as cosubstrate to enzymes like dehydrogenases containing the signatory sequence of -GXGGX- known as 'Rossman fold' [34]. Since the protein content of the placental extract is insignificant and peptides usually do not bind to nucleotides, it was verified whether NADPH could react with proteins/peptides or amino acids nonspecifically under the conditions of sterilization applied during manufacture of the extract.

Proteins unrelated to placental origin such as BSA, ovalbumin and lysozyme were sterilized separately in presence of NADPH as

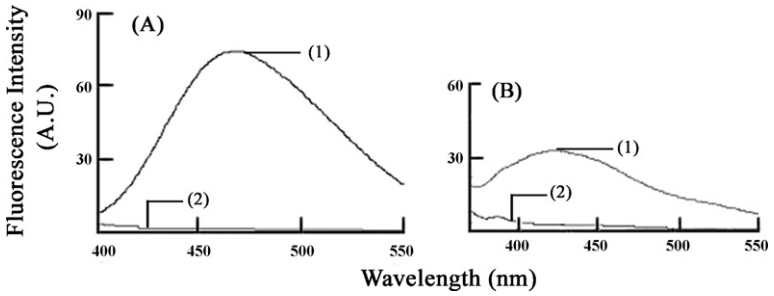

Fig. 5. Fluorescence emission spectra of (A) standard NADPH (42.0 μM) (1), after addition of 11.8 U glutathione reductase whereby 99% of fluorescence intensity was educed (2); (B) placental extract (20× concentrated) corresponding to 23.00 μM of NADPH before (1) and after incubation with glutathione reductase (2). Loss of fluorescence intensity was ~95%. Excitation was at 340 nm.

stated earlier and passed through Sephadex G-15 'spin column' to remove unbound NADPH from the proteins. Emission spectrum of the solutions (ex: 340 nm; em: 350–500 nm) before and after 'spin column' was followed. BSA and ovalbumin showed high affinity towards NADPH as 85–95% of NADPH fluorescence were recovered in the eluent. Lysozyme, however, did not show any significant reaction with NADPH.

Nonspecific reactions of amino acids with NADPH under the stated conditions were followed by TLC using three solvent systems. The following representative amino acids were used, glutamic acid (acidic), histidine and arginine (basic), serine and threonine (OH-containing) and alanine and methionine (aliphatic and sulfur containing). R_f of standard NADPH irrespective of heat treatment was between 0.79 ± 0.02 in the solvents. In summary, migration of amino acid–NADPH conjugate produced after heat treatment was considerably higher ($R_f = 0.75 \pm 0.05$–0.38 ± 0.05) as compared to free amino acids (R_f 0.05 ± 0.05). This indicates that the amino acids conjugates have polarity different from the free amino acids and were comparable more to NADPH.

3.7. Proteolysis of placental peptides

Placental peptides were digested with pronase to identify the linkage between the peptides and NADPH. The digest was fractionated using a C_{18} RP-HPLC column and the elution was followed at 340 nm to detect NADPH conjugates (Fig. 6). The chromatogram lead to partial separation of at least nine peaks with retention times 7.46 ± 0.03, 7.79 ± 0.03, 8.39 ± 0.02, 9.28 ± 0.03, 10.29 ± 0.04, 10.64 ± 0.03, 12.90 ± 0.04, 14.93 ± 0.03 and 19.53 ± 0.08 min respectively ($n = 4$). Standard NADPH was eluted at 14.96 ± 0.06 min close to peak no. 8.

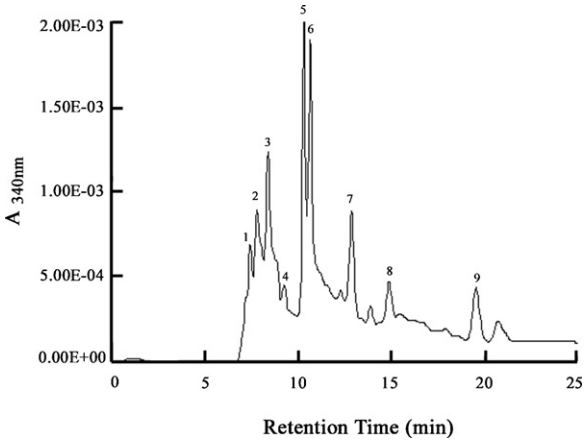

Fig. 6. Reverse phase HPLC profile of pronase digested placental peptides under isocratic condition of elusion with 0.01 M K-Phosphate, pH 7.0. Flow rate was 1 ml/min and the chromatogram was followed at $A_{340\,nm}$. Nine fractions as indicated were collected and analyzed by mass spectrometry.

3.8. Mass analysis

Mass spectrometric analysis was used to understand if the binding between NADPH and the peptides involved the functional nicotinamide moiety and to which amino acids the nucleotide was attached. Standard NADPH [M = NADPH, $4Na^+$] showed MS (FAB): m/z corresponding to ($M + H^+$) (observed: 834.22; expected: 834.35) and ($M + Na^+$) (observed: 856.23; expected: 857.35). The parent ions of NADPH were absent in NADPH–peptide conjugates. However, mass fragmentation patterns of bound NADPH showed ion species similar to that of standard NADPH. Fragmentation pattern of NADPH corresponded to that of NADPH–peptide conjugate (within parenthesis) having m/z of 382.13 (382.94), 266.93 (266.95), 202.23 (202.23), 164.97 (164.96), 128.99 (128.98) and 122.12 (122.13), which were derived from the nicotinamide end of the molecule. Among these, 382.94 (nicotinamide moiety + sugar + phosphate) and 122.12 (nicotinamide moiety) were of particular importance because they indicate intactness of the nicotinamide moiety.

Peaks isolated in the RP-HPLC were also subjected to mass analysis from which NADPH bound to glycine, proline, valine, leucine, isoleucine, aspartic acid, histidine and arginine were identified. NADPH bound to a dipeptide (pro, val) was also detected. Further, mass signals of free NADPH either protoned or deprotonated or attached with a sodium ion were also identified, which were probably produced as a result of ionization of the amino acid conjugates in mass analysis. The results have been summarized in Table 2. A representative profile of mass spectrometric analysis of peak 1 of the HPLC chromatogram has been shown in Fig. 7. The peaks

Table 2
Mass spectrometric analysis of placental-peptide and assignment of different fragments[a].

Fragment assigned/NADPH derivative	Mass	
	Observed	Calculated
(a) [NADPH] + ($-H^+$)	832.26	833.34
(b) [NADPH] + (H^+)	834.26	834.35
(c) [NADPH] + (Na^+) + ($-2H^+$)	854.13	854.35
(d) [NADPH·Gly] + ($-H_2O$) + ($-H^+$)	892.13	892.23
(e) [NADPH·Pro] + ($-H_2O$)	912.22	912.45
(f) [NADPH·Val] + ($-H_2O$)	914.22	914.45
(g) [NADPH·Leu/Ile] + (H^+) + ($-H_2O$)	930.11	929.55
(h) [NADPH·Asp] + ($-H_2O$)	930.21	930.45
(i) [NADPH·His] + (H^+) + ($-H_2O$)	952.15	952.45
(j) [NADPH·Arg] + ($-H_2O$)	972.15	972.55
(k) [NADPH·Pro·Val] + ($-H_2O$) + ($-H^+$)	992.01	992.55

[a] The observed m/z values of identical components in different fractions sometimes differ at decimal places.

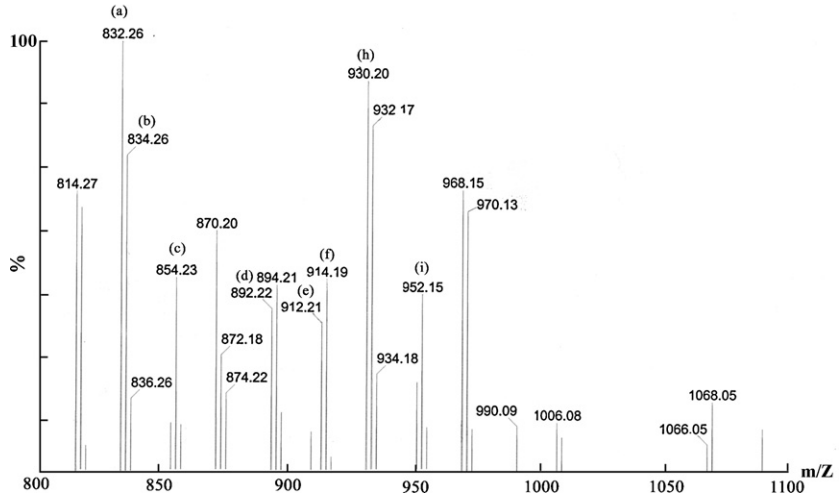

Fig. 7. Mass spectrometric analysis of peak 1 of Fig. 6. Origin of the peaks marked by alphabets has been described in Table 2.

corresponding to (a)–(c) or their combination were compulsorily present in all the chromatographic fractions as they were derived from NADPH. This data shows existence of the components (d)–(i) as described in Table 2. Multiple NADPH conjugates appear to be present because of partial overlapping of peaks 1–3 together with unresolved components.

4. Discussion

Binding of proteins and peptides to ligands provides an understanding as to how most of the biological processes occur within living systems. These interactions have specific consequences. Here it has been demonstrated using spectroscopic studies, immunoaffinity chromatography and mass spectrometric analysis that peptides isolated from human placental extract contains covalently bound NADPH. Nonspecific reaction between the two at elevated temperature and pressure required for sterilization of the product appears to be the cause as NADPH under similar conditions reacts irreversibly with proteins and amino acids with variable extent. The hydroxyl group or the sulfhydryl group of the amino acid residues in peptides and the phosphate group of the nucleotides are often the sites of linkage [35,36]. Other examples of covalent binding between peptides and nucleotides have also been reported elsewhere [37,38]

The extent of free and bound NADPH and peptides in the extract were analyzed holding two assumptions. First, the fluorescence (ex: 340 nm; em: 400–500 nm) of the extract comes exclusively from NADPH [20] and second, the peptides are the only proteinous component of the extract [19]. Based on these, it was estimated that $75.1 \pm 2.2\%$ of the nucleotide remains as free or bound to amino acids or very small peptides, while the rest remains bound to peptides. Conversely, it was estimated that 69.5% of the peptides remain free from NADPH (Table 1). Analysis of the immuno-affinity chromatogram corroborates these results (Fig. 4). In NADPH, it is the reduced nicotinamide nucleus that is responsible for fluorescence. Thus, the link between NADPH and the peptide spares the nicotinamide moiety. Mass spectrometric analysis of the peptides confirmed existence of nicotinamide in it. Spectrofluorimetric estimation of NADPH of the extract using glutathione reductase showed that the total NADPH content could be oxidized to NADP. This spectrofluorimetric analysis has been emphasized, as it is fluorophore specific and very sensitive. Spectrophotometric analysis of oxidoreductases based on NADP–NADPH conversion is less reliable in this case because of background absorption from impurities of the extract.

It is known from literature that topical application of fibronectin, a peptide component of the extract [39,40] and NADPH [41,42] is beneficial for wound healing. Recently presence of other peptides in the extract that are potentially active for wound healing has also been confirmed (D. De, unpublished results). The major part of the NADPH pool that remains free can be easily accessed by the cells for signaling and wound healing purpose. Nevertheless the bound form is also biologically active. It may act as a healing component even after deposition of the peptides at the site of the wound to enhance the healing process, thereby strengthening the wound healing potency of the extract. Further, spectrofluorimetric studies like FRET analysis of the peptides would not require additional labeling of fluorophore. The binding of NADPH also explains certain anomalous spectral behavior of the peptides.

5. Conclusion

Human placental extract contains $75.1 \pm 2.2\%$ free and the remaining as peptide bound NADPH that have been separated by molecular sieve and immuno-affinity chromatography. The nature of this binding is covalent. Spectrofluorimetric and mass spectrometric analysis along with assay using glutathione reductase indicate that the nicotinamide moiety of the total NADPH in the extract is biologically functional and thus available for cellular process as well as wound healing.

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